

Na⁺/H⁺ exchanger-dependent intracellular alkalization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes

STEPHAN J. RESHKIN, ANTONIA BELLIZZI, SANDRA CALDEIRA,*
VALENTINA ALBARANI,* ILARIA MALANCHI,* MANUELA POIGNEE,*
MARIANNA ALUNNI-FABBRONI,* VALERIA CASAVOLA, AND MASSIMO TOMMASINO*¹

Department of General and Environmental Physiology, University of Bari, Bari, Italy and

*Angewandte Tumorstudiologie, Deutsches Krebsforschungszentrum, Heidelberg, Germany

ABSTRACT In this study we investigate the mechanism of intracellular pH change and its role in malignant transformation using the E7 oncogene of human papillomavirus type 16 (HPV16). Infecting NIH3T3 cells with recombinant retroviruses expressing the HPV16 E7 or a transformation deficient mutant we show that alkalization is transformation specific. In NIH3T3 cells in which transformation can be turned on and followed by induction of the HPV16 E7 oncogene expression, we demonstrate that cytoplasmic alkalization is an early event and was driven by stimulation of Na⁺/H⁺ exchanger activity via an increase in the affinity of the intracellular NHE-1 proton regulatory site. Annulment of the E7-induced cytoplasmic alkalization by specific inhibition of the NHE-1, acidification of culture medium, or clamping the pHi to nontransformed levels prevented the development of later transformed phenotypes such as increased growth rate, serum-independent growth, anchorage-independent growth, and glycolytic metabolism. These findings were verified in human keratinocytes (HPK1A), the natural host of HPV. Results from both NIH3T3 and HPK1A cells show that alkalization acts on pathways that are independent of the E2F-mediated transcriptional activation of cell cycle regulator genes. Moreover, we show that the transformation-dependent increase in proliferation is independent of the concomitant stimulation of glycolysis. Finally, treatment of nude mice with the specific inhibitor of NHE-1, DMA, delayed the development of HPV16-keratinocyte tumors. Our data confirm that activation of the NHE-1 and resulting cellular alkalization is a key mechanism in oncogenic transformation and is necessary for the development and maintenance of the transformed phenotype.—Reshkin, S. J., Bellizzi, A., Caldeira, S., Albarani, V., Malanchi, I., Poignee, M., Alunni-Fabbroni, M., Casavola, V.,

Tommasino, M. Na⁺/H⁺ exchanger-dependent intracellular alkalization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes. *FASEB J.* 14, 2185–2197 (2000)

Key Words: cytoplasmic alkalization · NHE-1 · HPV16 E7 oncoprotein

TUMORS ORIGINATE FROM many different genetic alterations, which lead to the loss of normal growth control. Despite the genetic variability, two phenotypes common to all tumor cells are cellular alkalization and a shift to glycolytic metabolism. This cytoplasmic alkalization is the consequence of the stimulation of a member of the Na⁺/H⁺ exchanger protein family, NHE-1, a ubiquitously expressed transporter in the plasma membrane with a main function to extrude H⁺ from the cytoplasm (reviewed in refs 1, 2). Several studies support the concept that proliferation and transformation are associated with an increased intracellular pH (pHi). Rapid stimulation of the Na⁺/H⁺ exchanger, with consequent cytoplasmic alkalization, appears to be a universal response of quiescent cells to growth-promoting factors (2). Microinjection of activated ras p21 into quiescent mouse fibroblasts (NIH3T3) results in a transient morphological transformation, cell proliferation, and a rapid rise in pHi, whereas microinjection of the unaltered proto-oncogene p21 fails to produce any response (3). In agreement with the ras microinjection data, long-term expression of

¹ Correspondence: Deutsches Krebsforschungszentrum, Angewandte Tumorstudiologie, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany. E-mail: M.Tommasino@DKFZ-Heidelberg.de

oncogenic ras also leads to an intracellular alkalinization even when bicarbonate-dependent transport systems are activated by the presence of bicarbonate in the culture medium (4). Moreover, Na⁺/H⁺ exchanger-deficient cell lines either do not induce tumor formation or show severely retarded tumor growth when implanted in immuno-deprived mice (5). By an alternative approach, Perona and co-workers provided additional evidence for the importance of pHi in cellular transformation. Expression of the gene for the yeast plasma membrane H⁺-ATPase in immortalized rodent fibroblasts leads to a tumorigenic transformation (6) and a rise in pHi (7). Although these findings demonstrate an association of cytoplasmic alkalinization with tumorigenic transformation, it is not clear yet whether the rise of pHi plays a key role in the induction of cellular events that lead to transformation or is itself a consequent event of transformation (reviewed in refs 8, 9). Furthermore, the causal relationship between the shift to glycolytic metabolism and the increases in pHi and proliferation remains unclear.

To better characterize the role and mechanism of the Na⁺/H⁺ exchanger in transformation-dependent intracellular alkalinization and to understand whether it is an essential and early event in malignant transformation, we have developed an experimental cell model using the E7 oncoprotein of the human papillomavirus type 16 (HPV16). HPV16 E7 protein is able to induce tumorigenic transformation of mouse fibroblasts and to cooperate with another viral oncoprotein, E6, to immortalize primary human keratinocytes (reviewed in ref 10). These activities are in part explained by the ability of E7 to interact with the so-called 'pocket' proteins pRb, p107, and p130 (11–13). The pocket proteins are central regulators of cell cycle division. They negatively regulate, via direct association, the activity of several transcription factors, including members of the E2F family (14). Under normal cell cycle regulation, phosphorylation of pRb, which is mediated by cyclin-dependent kinase (CDK) activity, leads to the disruption of pRb/E2F inactive complexes. HPV16 E7 binds the pocket proteins and, analogous to the CDK-mediated phosphorylation, releases active E2Fs, which in turn positively regulate transcription of a group of genes encoding proteins essential for S-phase progression (reviewed in ref 15). The association of intracellular alkalinization with E7-driven transformation or the role of a possible alkalinization in the E7-dependent events has not yet been determined. Using the NIH3T3 cell line, in which transformation can be induced and followed by transcriptional activation of the HPV16 E7 oncogene, we demonstrate that cytoplasmic alkalinization is an early event in tumorigenic transformation and is driven by a stimulation of NHE-1 activity via an

increase in the affinity of the NHE-1 proton regulatory site. Most important, annulment of the E7-induced cytoplasmic alkalinization prevents the development of the transformed phenotype. Similar data were obtained in human keratinocytes, the natural host of the virus, which are immortalized by expression of the HPV16 E6 and E7 oncogenes. Last, treatment of nude mice with the amiloride analog 5-(N,N-dimethyl) amiloride (DMA), a specific inhibitor of NHE-1, delays the development of HPV16-keratinocyte tumors. Together our findings show that activation of the NHE-1 and resulting cellular alkalinization play a key role in oncogenic transformation.

MATERIALS AND METHODS

Cell culture manipulation

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose (4500 mg/l) supplemented with NaHCO₃ (3700 mg/l), 10% (v/v) heat-inactivated fetal bovine serum, L-glutamine (2 mM), Na-pyruvate (1 mg/ml), and 1 μ M tetracycline (only in the case of 2BN11 cells).

NIH3T3 cells constitutively expressing E7 genes were generated by infection with recombinant retroviruses (pBabe-puro vector (16) expressing HPV16 HA-tagged E7 proteins, wild-type or mutant. Cells were then selected in presence of 2.5 μ g/ml of puromycin. Two days later, puromycin-resistant colonies were pooled and used for the experiments.

The 2BN11 cell line was created by infecting NIH3T3 cells with recombinant retrovirus expressing the HPV16 E7 gene under the control of a tetracycline repressed promoter (17). After selection in puromycin, the final clone (2BN11) was selected on the basis of having undetectable basal E7 expression in the presence of tetracycline and high inducibility as determined by Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Partial inhibition of glycolysis was obtained by culturing the cells for 6 h in DMEM medium high glucose (4500 mg/l) supplemented with increasing concentrations of deoxyglucose (0.1–0.5 mg/ml).

RNA preparation and RT-PCR

2BN11 total RNA was prepared using StrataPrep Total RNA Miniprep Kit (Stratagene, San Diego, Calif.). The first strand cDNA was synthesized using 1 μ g of total RNA and a commercially available kit (MBI, Hanover, Md.). The E7 and GAPDH fragments were amplified by PCR using specific primers and 3 μ l of each cDNA sample as template.

Determination of intracellular pH, intrinsic buffering capacity (β_1), and H⁺ efflux rates

Cytoplasmic pHi was measured spectrofluorimetrically with the fluorescent pH-sensitive probe, BCECF, trapped intracellularly in cell monolayers grown on plastic coverslips. Cells were loaded for 1 h at room temperature with the acetoxymethyl ester of BCECF (10 μ M) in 'Na⁺ ringer'. After loading, coverslips with confluent monolayers were inserted at an angle of 60° in a fluorometer cuvette designed to permit easy solution change with multiple perfusion solutions as de-

scribed previously (18). Fluorescence was monitored in a Shimadzu RF 5000 spectrofluorometer using alternately 455 nm (pH insensitive) and 490 nm (pH-sensitive) as excitation wavelengths with a xenon light source (5 nm bandwidth). Emission was measured at 530 nm (15 nm bandwidth). pHi was calculated from the fluorescence emission ratio of the two excitation wavelengths using a standard calibration procedure based on the use of nigericin (0.5 μ M) in high-potassium K clamp ringer (19). The activity of the Na^+/H^+ exchanger was measured by monitoring pHi recovery after an intracellular acid load produced with the NH_4Cl prepulse technique (20). The rate of Na^+ -dependent alkalization was determined by linear regression analysis of 15 points taken at 4 s intervals. The use of $\text{CO}_2/\text{HCO}_3^-$ -free solutions minimizes the likelihood that Na^+ -dependent HCO_3^- transport was responsible for the observed pHi changes.

The pHi dependence of intracellular buffer capacity was computed by the NH_4 pulse method (21). β_i refers to the ability of intrinsic cellular components, excluding HCO_3^- , CO_2 , to buffer changes in pHi and is defined as the moles of H^+ required to produce a one-unit shift in pHi per liter of intracellular fluid. The actual activity of the exchanger in terms of proton flux rate (mM/min) is determined by multiplying the rates in pHi change by the cells intrinsic buffering capacity (β_i) at the pHi in which the measurement was being made. The pHi dependence of total net proton extrusion rate was calculated from the data of pHi recovery curves as described (22).

Equilibration of external and internal pH with tributyltin bromide

The pHi of the cells was clamped by incubation with 5 μ M tributyltin bromide (Aldrich, Milwaukee, Wis.) in DMEM at a medium pH of either 6.9 or 7.4 with Na-HEPES buffer (23). Initial experiments indicated that the pHex and pHi are rapidly equilibrated in the presence of tributyltin bromide (data not shown).

Determination of cellular parameters

Proliferation was measured in each sample by determining cell number as described previously (24) and by measuring the incorporation of bromouridine following either the Cell Proliferation ELISA colorimetric assay or the BrdU Labeling and Detection kit I according to the manufacturer instructions (Boehringer Mannheim, Mannheim, Germany). Cells were labeled for 3–6 h with BrdU.

The ability to grow in an anchorage-independent way was measured by the formation of colonies on soft agar. The base layer was prepared with DMEM:0.5% low melting temperature agarose (SeaPlaque, FMC BioProducts, Rockland, Maine). The feed layer was then prepared by resuspending 5000 cells in DMEM:0.25% low melting temperature agarose plus experimental compounds and pouring onto the previously prepared base layer (1/2 of the volume of the lower layer). Culture medium was changed every 2 days and colonies counted after 21 days.

To measure lactate production, cell monolayers were incubated for 10 min in a buffer without glucose (in 110 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 4 mM Na_2PO_4 , 50 mM Na-HEPES, pH 7.4). The cells were then washed twice in the same buffer and glycolysis was initiated by addition of 20 mM glucose (23). The concentration of lactate released into the buffer was measured either by an enzyme-linked assay system as described (25) or with a colorimetric kit supplied by Sigma (St. Louis, Mo.).

Determination of cyclin E promoter activity

Activation of the cyclin E promoter by HPV16 E7 was determined in NIH3T3. Cells were transfected by calcium phosphate precipitation as described elsewhere (26). At 16 h after transfection, cells were cultured in DMEM containing 0.5% fetal calf serum (FCS) for 24 h in absence or presence of 5 μ M DMA. Protein extracts were prepared and luciferase and β -galactosidase assays performed. Details of the construct containing the cyclin E promoter are given elsewhere (27).

Protein extraction and Western blotting

For the detection of HPV16 E7, β -tubulin, cyclins E and A, and NHE-1, total cellular extracts were prepared as described in ref 28, 29. 100 micrograms of total extract was precipitated in acetone (9:1/v/v 20 min at -20°C , centrifuged (12,000 g, 10 min) and resuspended in 20 μ l of gel loading buffer. Total cell extracts were fractionated by electrophoresis on a polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). Proteins were transferred onto a Polyscreen PVDF transfer membrane (DuPont, Wilmington, Del.) in a Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad, Hercules, Calif.). Immunocomplexes were detected with ECL reagent (Amersham, Little Chalfont, U.K.). We used the following antibodies: anti HA-tag monoclonal antibody (MMS-101R BabCo, dilution 1:1000), anti HPV16 E7 monoclonal antibody (Triton, dilution 1:200), anti-human cyclin E antibody (14761C PharMingen; dilution 1:1000), anti-human cyclin A antibody (H-432 Santa Cruz Biotechnology; dilution 1:1000), anti-human p34CDK2 antibody (kindly provided by Dr. M. Pagano, New York University Medical Center, New York; dilution 1:2000), anti β -tubulin monoclonal antibody (Tub 2.1 Sigma; dilution 1:1000), anti NHE-1 antibody (kindly provided by Dr. Pouyssegur, CNRS, Nice, France; dilution 1:1000).

Generation of tumors in nude mice and treatment protocol

Swiss athymic CD-1 (nu/nu) nude mice weighting 20–25 g were injected subcutaneously on the back with HPKIA cells (10^7 cells in 100 μ l phosphate buffer). Half the mice were treated every other day by intraperitoneal (i.p.) injections of 15 μ g DMA/gm body weight whereas the other half received only the vehicle (4% DMSO in sterile water). Tumor length, width, and height were measured with calipers the third day after injection and then weekly.

RESULTS

Only transformation competent HPV16 E7 induces cellular alkalization

Our goal was to study the mechanism and significance of the increase in intracellular pH in transformation driven by a biologically relevant oncogene. The E7 oncoprotein of HPV16 is known to induce transformation both *in vitro* and *in vivo* and to fully transform immortalized rodent fibroblasts (E7/NIH3T3 cells), which have been shown to be tumorigenic in nude mice (10). To determine whether E7-dependent transformation leads to a cytoplasmic alkalization, immortalized NIH3T3 cells were infected with recombinant retroviruses expressing ei-

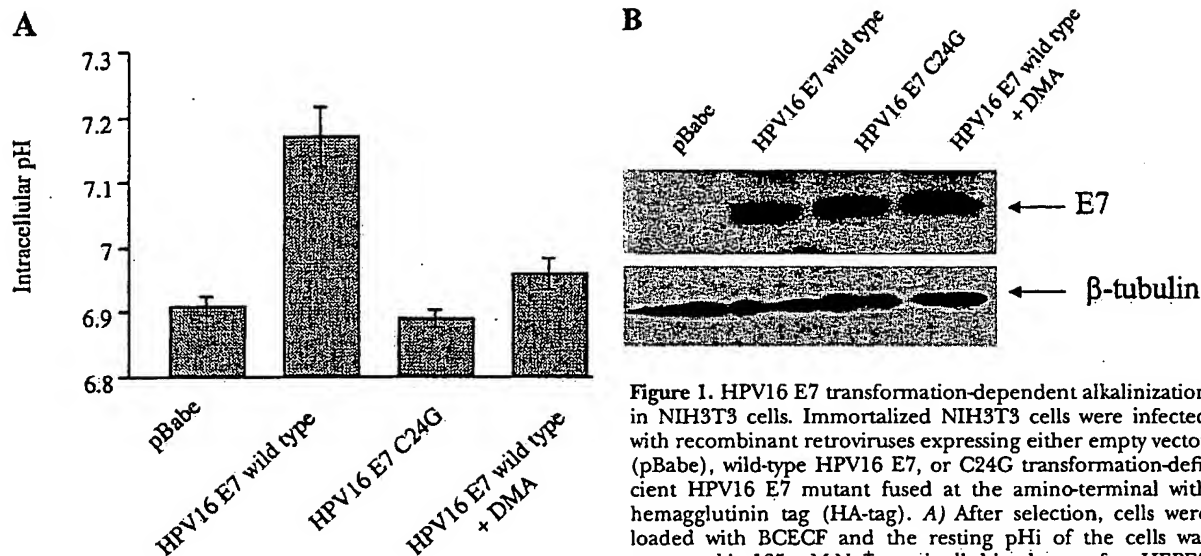


Figure 1. HPV16 E7 transformation-dependent alkalinization in NIH3T3 cells. Immortalized NIH3T3 cells were infected with recombinant retroviruses expressing either empty vector (pBabe), wild-type HPV16 E7, or C24G transformation-deficient HPV16 E7 mutant fused at the amino-terminal with hemagglutinin tag (HA-tag). **A**) After selection, cells were loaded with BCECF and the resting pH_i of the cells was measured in 135 mM Na⁺ nominally bicarbonate-free HEPES buffer (pH 7.4) as described in Materials and Methods.

Intracellular pH was measured in cells expressing wild-type HPV16 E7 in the absence or presence of 5 μM DMA. Bars are mean ± SE, n=4. **B**) The levels of HPV16 E7 proteins in NIH3T3 cells were determined by immunoblotting. 100 μg of total protein extracts were applied on 12% polyacrylamide-SDS gel, transferred to PVDF membranes and incubated with anti-HA-tag or β-tubulin antibodies to determine the levels of HPV16 E7 and β-tubulin, respectively. The β-tubulin signal was used as loading control.

ther HPV16 E7 wild-type or a transformation-deficient mutant (30, 31). The effect of E7 expression on steady-state pH_i was then determined by spectrofluorometry as described in Materials and Methods. As seen in Fig. 1A, the wild-type protein elevated resting pH_i by ~0.25 units, whereas the transformation-negative E7 mutant was not able to induce alkalinization. Addition of DMA, a specific inhibitor of the Na⁺/H⁺ exchanger isoform 1 (NHE-1), to the culture medium abolished the E7-induced alkalinization. Western analysis (Fig. 1B) showed that the inability of the E7 mutant to alkalinize the cells was not due to different expression levels and DMA treatment did not decrease the E7 protein expression. These results are consistent with the reported specificity of only oncogenic and not proto-oncogenic ras in inducing intracellular alkalinization in NIH3T3 cells (3).

E7-dependent alkalinization is an early event and drives subsequent glycolysis

The above experimental system does not permit determination of the dynamic processes occurring during transformation. To address these issues, a cell model was constructed in which the transformation of normal cells could be rapidly induced and early events subsequently monitored. NIH3T3 cells were infected with a recombinant retrovirus in which HPV16 E7 gene expression is under the control of a promoter that is negatively regulated by tetracycline

(17), clone 2BN11. As can be seen in Fig. 2, in 2BN11 cells HPV16 E7 can be detected only after tetracycline removal. The HPV16 E7 protein was present at lower levels in 2BN11 cells than in CaSki, an HPV16-positive cell line derived from human cervical cancer (Fig. 2). The development of transformed phenotypes (e.g., increased proliferative rate, anchorage-independent growth, serum independence, and increased glycolytic metabolism) are

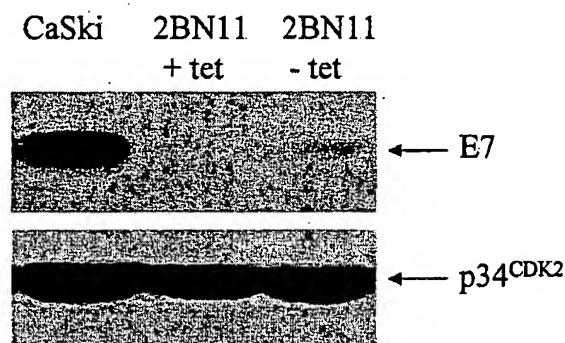


Figure 2. HPV16 E7 expression in 2BN11 cells after tetracycline removal from the culture medium. Total proteins were isolated and Western analysis performed with anti-HPV16 E7 or p34^{CDK2} antibodies as described in Materials and Methods. Lane 1: control HPV16 E7 expression in a human cervical cancer cell line (CaSki); lane 2: 2BN11 cells cultured in the presence of 1 μM tetracycline; lane 3: 2BN11 cells 24 h after tetracycline removal from the culture medium. The p34^{CDK2} signal was used as loading control.

under the strict control of E7 expression in 2BN11 cells (data not shown).

The effect of E7 expression on steady-state pHi in 2BN11 cells was then determined. E7-mediated transformation was induced by removing tetracycline from the culture medium for 24 h and the resting pHi was determined by spectrofluorometry. In 2BN11 cells, resting pHi increased with E7-induced transformation from 6.89 ± 0.017 to 7.16 ± 0.019 ($n=25$, $P<0.001$). The same shift in pHi was observed in cells cultured in the presence of bicarbonate (data not shown). Nontransfected NIH3T3 cells have a similar resting pHi as 2BN11 cells, which do not express E7 (6.91 ± 0.019 , $n=5$, $P>0.5$), and addition or removal of tetracycline had no effect on this value (data not shown). The alkalinization in transformed 2BN11 cells was blocked by addition of $5 \mu\text{M}$ of DMA (6.86 ± 0.012 , $n=6$), whereas $10 \mu\text{M}$ of the bicarbonate transporter inhibitor, 4,4'-diisothiocyanostilbene-2, 2'-disulfonic acid, had no effect on the pHi (7.19 ± 0.015 , $n=5$). To confirm that the variation of pHi was due the inhibition of NHE-1 and

not to an unspecific effect of DMA or to the inhibition of other isoform of NHE, we used another specific inhibitor of the NHE-1 isoform, HOE 694 (reviewed in refs 1, 2). Similar results were obtained with NHE-1 inhibitors DMA and HOE 694 (data not shown). These data, together with those shown in Fig. 1, demonstrate that cellular alkalinization is associated with E7-induced transformation and is dependent on the Na^+/H^+ exchanger.

To determine whether alkalinization is an early event in transformation, the temporal sequence of the change in resting pHi was monitored over a time course before and after induction of E7 expression. Monolayers loaded with the pHi-sensitive fluorescent indicator were first perfused with Na^+ ringer plus $1 \mu\text{M}$ tetracycline. In these conditions, resting pHi (6.88 ± 0.014 , $n=9$) remained constant for as long as 5 h (data not shown). Figure 3A shows a representative experiment of the time course of changes in resting pHi in perfused monolayers. After tetracycline removal, resting pHi remained constant for ~ 90 min and then started a slow, regular rise that

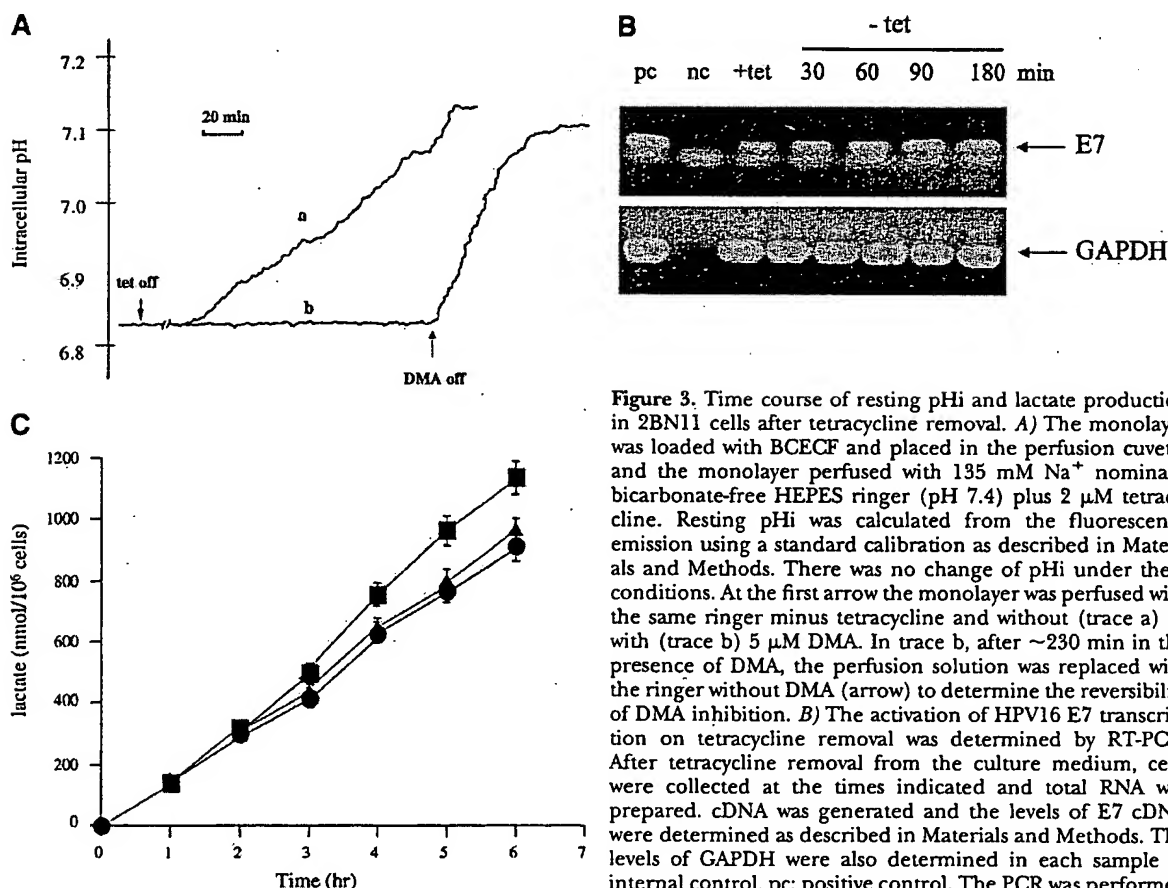


Figure 3. Time course of resting pHi and lactate production in 2BN11 cells after tetracycline removal. **A)** The monolayer was loaded with BCECF and placed in the perfusion cuvette and the monolayer perfused with 135 mM Na^+ nominally bicarbonate-free HEPES ringer (pH 7.4) plus $2 \mu\text{M}$ tetracycline. Resting pHi was calculated from the fluorescence emission using a standard calibration as described in Materials and Methods. There was no change of pHi under these conditions. At the first arrow the monolayer was perfused with the same ringer minus tetracycline and without (trace a) or with (trace b) $5 \mu\text{M}$ DMA. In trace b, after ~ 230 min in the presence of DMA, the perfusion solution was replaced with the ringer without DMA (arrow) to determine the reversibility of DMA inhibition. **B)** The activation of HPV16 E7 transcription on tetracycline removal was determined by RT-PCR. After tetracycline removal from the culture medium, cells were collected at the times indicated and total RNA was prepared. cDNA was generated and the levels of E7 cDNA were determined as described in Materials and Methods. The levels of GAPDH were also determined in each sample as internal control. pc: positive control. The PCR was performed using as template 10 (8) copies of a plasmid containing the E7

or the GAPDH gene. nc: negative control. The PCR was performed in absence of a template. **C)** Determination of lactate production. At time zero, monolayers were incubated in growth medium plus tetracycline (circles), minus tetracycline (squares), or minus tetracycline plus $5 \mu\text{M}$ DMA (triangles). At the indicated times, lactate concentration was measured in the samples. Points are mean lactate concentration \pm SE, $n=3$.

continued until it reached a new constant value after 1.5 to 2 h, reflecting the new resting pHi (trace a). The mean time necessary for the initiation of alkalization after tetracycline removal was 1.4 ± 0.11 h ($n=9$) and was independent of the original perfusion time with tetracycline. The mean time to accomplish the alkalization after initiation was 1.8 ± 0.25 h ($n=9$). Trace b shows that the addition of DMA at the time of tetracycline removal completely inhibited the alkalization. As expected, since DMA inhibition is fully reversible (reviewed in refs 1, 2), its removal (arrow) resulted in a very rapid alkalization to the same elevated resting pHi as in the cells that had not been inhibited (trace a). Alkalization was concomitant with activation of E7 transcription after the removal of tetracycline. RT-PCR analysis showed that the levels of E7 mRNA rapidly increase when cells are cultured in absence of tetracycline (Fig. 3B). However, low levels of mRNA can also be detected in cells grown in medium containing tetracycline (Fig. 3B), but they are not sufficient to induce transformation. Indeed, 2BN11 cells have the same features of the parental cells, NIH3T3, when cultured in tetracycline-containing medium (data not shown). This rapid alkalization is similar to that reported for microinjection of high concentrations of oncogenic ras (3) and suggests that the cellular alterations necessary for the transformation-dependent alkalization were accumulating during this time but that alkalization could not take place due to the block of the exchanger. As glycolytic metabolism also increases with E7-driven transformation, it was important to determine the relationship between the activation of alkalization and glycolysis. Figure 3C shows that the transformation-dependent increase in lactate production (squares) started between 2 and 2.5 h after tetracycline removal and was inhibited by DMA (triangles). These data show that the shift to glycolytic metabolism is directly driven by the rise in pHi.

E7-dependent transformation results in an increase in the affinity of the intracellular NHE-1 proton regulatory site

As the above data indicate that the Na^+/H^+ exchanger is directly involved in the E7-dependent alkalization, we next elucidated the alterations of Na^+/H^+ exchanger kinetics during E7-induced transformation. We first determined the activity of the exchanger by measuring its rate in returning pHi to the resting value at two external sodium concentrations after an acid load (20). As illustrated in Fig. 4A, in the absence of sodium there was no recovery of pHi and addition of sodium (135 mM) to the cells produced a rapid rise in pHi (traces a), which was slower in 34 mM sodium (traces b). HPV16 E7-

expressing cells (-tet, 24 h) had an increased recovery rate at both 34 and 135 mM external sodium and returned to a higher resting pHi value than did the nontransformed (+tet) cells (6.85 ± 0.017 ($n=4$) vs. 7.10 ± 0.018 ($n=8$) for +tet and -tet treatments, respectively). Similar increases in Na^+/H^+ exchange-dependent recovery occurred at both short (3–6 h) and long (24–96 h) periods after E7 induction (data not shown). The response of intrinsic buffering capacity (β_i) to transformation was next determined and used to calculate absolute exchanger activity (21). The average calculated H^+ extrusion rate due to Na^+/H^+ exchanger activity significantly increased from 6.33 ± 1.38 in normal cells to 9.45 ± 1.81 and 12.24 ± 1.58 $\text{mM H}^+ \text{ min}^{-1}$ in cells, in which E7 expression was induced for 3 and 48 h, respectively ($n=12$, $P<0.001$). Western blot analysis showed that the increased H^+ extrusion rate was not due to a change in NHE-1 protein expression in either short- or long-term E7-induced transformation (Fig. 4B). This increase in initial transport rate without a corresponding increase in transporter protein expression is indicative of an increased transporter turnover rate (32). To determine whether the increased H^+ extrusion rate was a result of alterations in the NHE-1 proton regulatory site kinetics, the pHi dependence of the NHE-1 activity was analyzed (20). Figure 4C shows that 3 h of E7 induction shifted the curve representing the dependence of exchanger activity ($\text{mM H}^+/\text{min}$) on pHi to the right. This is indicative of an increase in the apparent affinity of the intracellular proton regulatory site of the exchanger (2). Longer periods of E7 expression produced only slightly more pronounced rightward alkaline shifts (data not shown).

Role of transformation-dependent alkalization in driving other transformed phenotypes

Intracellular pH has been shown to be an important regulator of a number of cellular processes such as cell shape, proliferation, apoptosis, and secretion of proteases (1, 2, 33–35). However, the role of the alkalization that occurs during transformation in the induction of anchorage-independent growth and in serum independence has not been clarified (reviewed in refs 8, 9). To characterize the role of early E7-dependent alkalization in the subsequent development of these transformed phenotypes, E7-expressing cells were cultured in the presence or absence of 5 μM DMA. In Fig. 5 it can be seen that the inhibition of the intracellular alkalization by DMA after tetracycline removal was able to decrease the growth rate to the level of nontransformed cells (Fig. 5A) and to suppress the development of serum-independence (Fig. 5B) and anchorage-independent growth (Fig. 5C). The lack of effect of DMA on

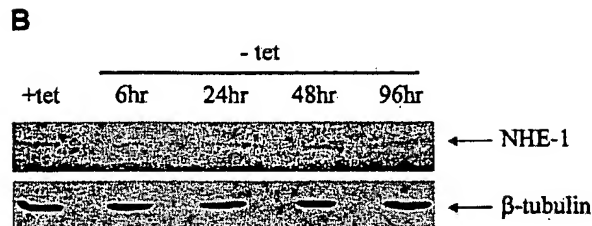
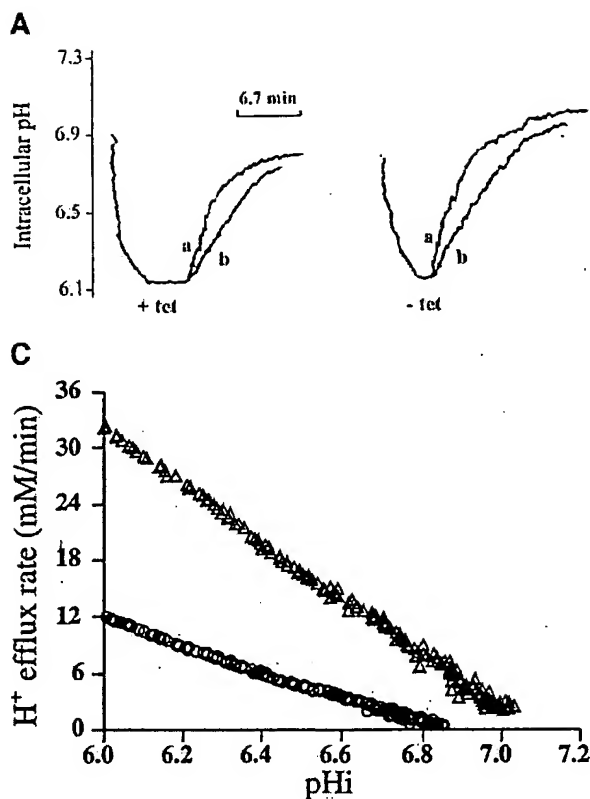


Figure 4. E7 expression stimulates NHE-1 activity by an increase in the affinity of the intracellular proton regulatory site of the exchanger without alterations in Na⁺/H⁺ exchanger expression. **A)** Transformation increases the rate of NHE-1-dependent recovery from an acid load and the resting pHi. All measurements were conducted in nominally HCO₃⁻ free, HEPES buffered solutions in order to measure only the Na⁺/H⁺ exchanger. BCECF-loaded cells initially perfused with bicarbonate-free Na⁺-ringer were exposed for 5 min to 20 mM NH₄Cl in the same ringer. This ringer was then replaced by a NH₄Cl-free, NMDA-Cl solution resulting in an acidification of the cytoplasm that remained stable in the absence of external sodium (the first part of each trace). When the monolayer was perfused with 135 mM Na⁺ nominally bicarbonate-free HEPES solution (pH 7.4), a rapid recovery of pHi commenced (traces a). The recovery of the first run is compared with the second run performed in the presence of 34 mM sodium (traces b). The recovery at these two sodium concentrations are shown for nontransformed cells (+tet) and transformed cells in which tetracycline had been removed for 24 h. **B)** Determination of NHE-1 levels by immunoblotting. After tetracycline removal from the culture

medium, cells were collected at the times indicated and total protein extracts were prepared. 100 μg of protein extracts were applied on 7.5% polyacrylamide-SDS gel, transferred to PVDF membranes, and incubated with anti NHE-1 or β-tubulin antibodies. The β-tubulin signal was used as loading control. **C)** Analysis of the function of the intracellular allosteric proton regulatory site in 2BN11 cells before and after tetracycline removal. Transformation produced an alkaline shift in the pHi dependence of total net proton extrusion rate in cells in which tetracycline had been removed for 3 h (triangles) compared to nontransformed (+tet) cells (circles). These plots were computed from experiments such as those illustrated in panel A.

basal growth is consistent with the hypothesis that the Na⁺/H⁺ exchanger and the rise in pHi have a permissive role in the initiation of proliferation without being involved in steady-state growth. To exclude the possibility that abrogation of E7-induced transformation was due to a nonspecific DMA effect, we reduced or annulled the E7-induced alkalization by culturing the cells in a growth medium having a pH of 6.9 in the absence or presence of the proton ionophore, tributyltin bromide (23). Lowering the external pH results in a drastically reduced pHi in 3T3 cells that is resistant to change by proton or bicarbonate transporters (7). Tributyltin bromide functions as an anion exchanger permitting the rapid equalization of pHi with medium pH and it is preferable to another common ionophore, nigericin, since it inserts only in the plasma membrane (23). As can be observed in Fig. 6A, the decrease of external pH to 6.9 was sufficient to reduce the E7-dependent increase in growth rate in DMEM containing 10% (Fig. 6A), whereas clamping pHi to 6.9 further increase this effect. Similarly, the same treatments—decrease of external pH or clamping

pHi to 6.9—negatively influence the E7-mediated stimulation of serum-independent growth (Fig. 6B) and lactate production (Fig. 6C). In contrast, clamping pHi to 7.4 further increased the levels of the transformed phenotypes (Fig. 6A–C). Together these data demonstrate that inhibition of alkalization is sufficient to suppress the development of the other transformed phenotypes.

Inhibition of serum-independent growth by DMA does not affect E7 induced cyclin E activation

The ability of HPV16 E7 to drive quiescent cells into S phase is in part mediated by neutralization of pRb function and consequent activation of E2F-regulated genes, e.g., cyclin E (15). Since the inhibition of cytoplasmic alkalization by DMA prevents E7-expressing cells to enter S phase, it was important to determine whether variations of intracellular pH influence the ability of HPV16 E7 to activate E2F-driven transcription. For this purpose, NIH3T3 cells were cotransfected with a construct expressing HPV16 E7 protein together with a vector containing

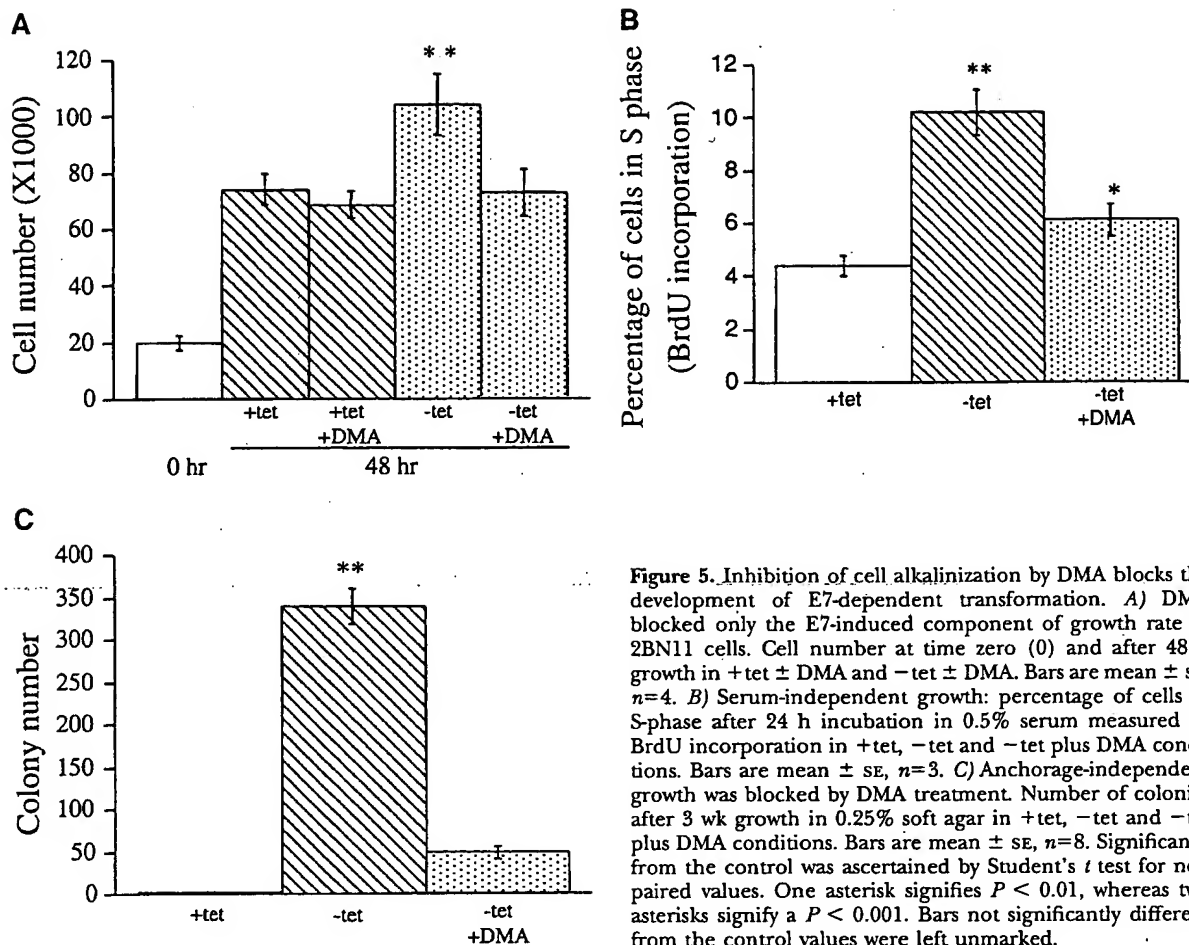


Figure 5. Inhibition of cell alkalization by DMA blocks the development of E7-dependent transformation. **A)** DMA blocked only the E7-induced component of growth rate of 2BN11 cells. Cell number at time zero (0) and after 48 h growth in +tet ± DMA and -tet ± DMA. Bars are mean ± SE, $n=4$. **B)** Serum-independent growth: percentage of cells in S-phase after 24 h incubation in 0.5% serum measured by BrdU incorporation in +tet, -tet and -tet plus DMA conditions. Bars are mean ± SE, $n=3$. **C)** Anchorage-independent growth was blocked by DMA treatment. Number of colonies after 3 wk growth in 0.25% soft agar in +tet, -tet and -tet plus DMA conditions. Bars are mean ± SE, $n=8$. Significance from the control was ascertained by Student's *t* test for non paired values. One asterisk signifies $P < 0.01$, whereas two asterisks signify a $P < 0.001$. Bars not significantly different from the control values were left unmarked.

the cyclin E promoter cloned in front of the luciferase gene (27). As shown in Fig. 7, the addition DMA to the culture medium had no effect on the E7-dependent transactivation of the cyclin E promoter. These data indicate that in the E7-mediated induction of serum-independent growth, alkalization must act on a pathway(s) parallel to the activation of E2F-driven transcription.

Alkalization in HPV16 transformed human primary keratinocytes drives other transformed phenotypes

To further confirm the role of cytoplasmic alkalization in HPV-induced transformation, we extended our study to human primary keratinocytes, the natural host of the virus. HPK1A cells are primary human keratinocytes immortalized by transfection of the entire HPV16 genome that become spontaneously transformed at high passage number (36). As shown in Table 1, the resting pH_i was higher in the transformed (late passage) keratinocytes than in the immortalized (early passage) cells. This alkaliza-

tion was linked to an increased NHE-1 activity and the addition of DMA reversibly reduced the resting pH_i to levels similar to those for the immortalized cells. The effect of DMA on growth rate and soft agar colony formation was next assessed (Fig. 8). The late passage cells had a higher growth rate (Fig. 8A, B) and a greater capacity for colony formation in soft agar (Fig. 8C) than early passage HPK1A. All these parameters were reduced to levels similar to those in the early passage cells by the addition of DMA and subsequent reduction of pH_i to normal levels (Fig. 8). These data are in full agreement with those obtained in the rodent fibroblast model and underline the conserved nature of the mechanism.

As the data in NIH3T3 cells indicate that E7-induced, serum-independent growth requires intracellular alkalization independently of the stimulation of E2F-driven transcription, we could predict that the expression of E2F-regulated genes would not differ between early and late passage HPK1A cells. Therefore, we determined the endogenous levels of cyclin E and cyclin A, which are encoded by E2F-regulated genes, in early and late passage

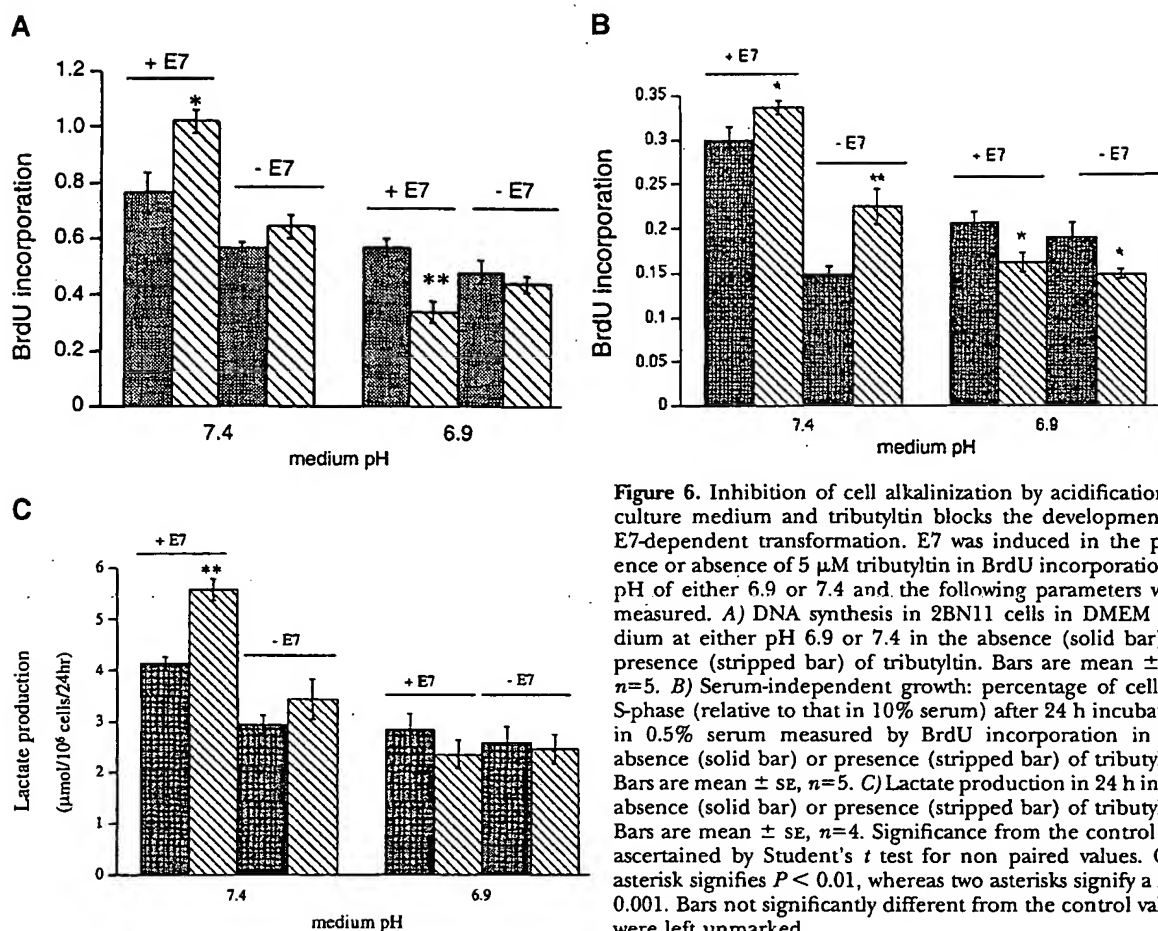


Figure 6. Inhibition of cell alkalization by acidification of culture medium and tributyltin blocks the development of E7-dependent transformation. E7 was induced in the presence or absence of 5 μ M tributyltin in BrdU incorporation at pH of either 6.9 or 7.4 and the following parameters were measured. **A)** DNA synthesis in 2BN11 cells in DMEM medium at either pH 6.9 or 7.4 in the absence (solid bar) or presence (stripped bar) of tributyltin. Bars are mean \pm SE, $n=5$. **B)** Serum-independent growth: percentage of cells in S-phase (relative to that in 10% serum) after 24 h incubation in 0.5% serum measured by BrdU incorporation in the absence (solid bar) or presence (stripped bar) of tributyltin. Bars are mean \pm SE, $n=5$. **C)** Lactate production in 24 h in the absence (solid bar) or presence (stripped bar) of tributyltin. Bars are mean \pm SE, $n=4$. Significance from the control was ascertained by Student's *t* test for non paired values. One asterisk signifies $P < 0.01$, whereas two asterisks signify a $P < 0.001$. Bars not significantly different from the control values were left unmarked.

HPKIA cells. As shown in Fig. 9, there was no significant difference in the expression of either protein in the early passage compared to the late passage cells cultured in the absence or presence of DMA. Similar results were obtained in the analysis of cyclin E and cyclin A transcripts by Northern blotting (data not shown). These data confirm that cytoplasmic alkalization and activation of E2F-regulated transcription act independently in driving transformation.

Activation of glycolysis does not contribute to the increased proliferation in transformed HPKIA cells

As in the 2BN11 cells, we observed an increase in glycolytic metabolism in the transformed HPKIA cells (late passage) that was reduced to levels measured in the early passage cells by DMA treatment (data not shown). We next determined whether the increased glycolytic metabolism plays a role in the stimulated growth rate associated with transformation in HPKIA cells. Glycolysis can be inhibited by incubation with deoxyglucose. We observed that addition of 0.1 mg/ml deoxyglucose to the culture

medium (containing 4.5 mg/ml glucose) decreases glycolysis to the levels of DMA-treated cells (Fig. 10). In these conditions we observed that the concomitant reduction in proliferation was only ~20% of that occurring when the alkalization was blocked with DMA. Since deoxyglucose can induce apoptosis, most likely due to the ATP deprivation, the percentage of apoptotic cells was also determined. No significant difference of cell death was observed when cells were grown in normal medium or at these concentrations of deoxyglucose (data not shown). On the basis of these findings we conclude that the pH_i-induced stimulation of glycolysis plays a minor role in the activation of proliferation during transformation.

DMA blocks tumor cell growth *in vivo*

Finally, the effect of the direct inhibition of NHE-1 activity on tumor initiation and growth was measured in an *in vivo* experimental system. Late passage HPKIA cells were injected subcutaneously in nude mice. Half of the mice were treated every other day by i.p. injections of 15 μ g DMA/gm body weight

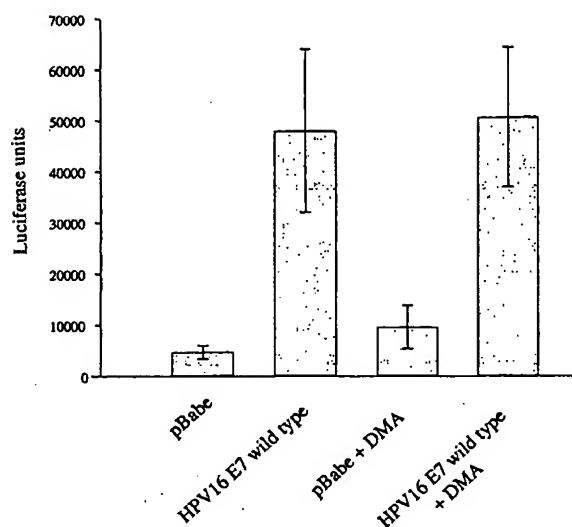


Figure 7. Variation of intracellular pH does not influence the HPV16 E7 ability to transactivate E2F-driven transcription. The ability of HPV16 E7 protein to transactivate the murine cyclin E promoter was assayed by transient cotransfection of NIH3T3 cells cultured in absence or presence of 5 μ M DMA. The different pBabe-puro vectors were cotransfected with a construct containing cyclin E promoter cloned in front of the luciferase gene. Luciferase activity was normalized to β -galactosidase activity obtained from a cotransfected cytomegalovirus-driven β -galactosidase plasmid. The data are the mean of two independent experiments.

whereas the other half received only the vehicle. Tumor size was measured the third day after injection and then at weekly intervals. As can be seen in Fig. 11, DMA treatment retarded tumor development. These data are consistent with results from independent *in vivo* studies in which tumors were induced with different cancer cell types (37, 38).

DISCUSSION

The association between oncogenic transformation and intracellular alkalinization is well documented, but there are conflicting data concerning whether the increase in pHi is an early event in transformation and whether it is directly involved in determining the development of the new cellular state. Furthermore, it is not yet completely clear whether the

alkalinization is driven by an activation of the Na^+/H^+ exchanger or by the shift toward glycolytic metabolism. In this study, we have chosen an experimental model where cellular transformation is induced by the E7 oncoprotein of the malignant human papillomavirus type 16. The use of an inducible expression system for E7, which permitted a finely controlled study of the sequence of early events occurring during transformation and the recognition of causal interactions required for transformation, allowed us to demonstrate that alkalinization is an early event in oncogenic transformation. Moreover, these data demonstrated that, as shown for other oncoproteins, E7-induced transformation is associated with intracellular alkalinization. Transformation-deficient E7 mutant is not able to induce alkalinization, demonstrating that the alkalinization is, in fact, a transformation-associated phenomenon. This observation is in line with the demonstration that ras oncoprotein injection induces a rapid alkalinization in NIH3T3 cells, whereas injection of proto-oncogene did not (3). The alkalinization is driven by the activation of NHE-1, as inhibition of NHE-1 activity by its specific inhibitor, DMA, results in the abrogation of the increased pH. The stimulation of Na^+/H^+ exchanger activity occurs via an increase in the affinity of the intracellular proton regulatory site of the exchanger and is not dependent on increased Na^+/H^+ exchanger expression. This produces an alkaline shift of the relationship between the rate of the NHE-1 and pHi that resembles the mechanism involved in activation of the exchanger by serum, growth factors, and hormones (2).

Most important, our data show that the alkalinization is a key event for the establishment and maintenance of oncogenic transformation. Upon expression of the HPV16 E7 oncoprotein, the cells develop a series of phenotypes characteristic of neoplastic transformation: increased growth rate, anchorage-independent growth, serum-independent growth, increased glycolysis, and cytoplasmic alkalinization. Specific inhibition of NHE-1 activity by DMA prevented the development of the transformation-associated increase in growth rate, serum independence of growth, anchorage-independent growth and glycolytic metabolism, and *in vivo* tumor development

TABLE 1. Intracellular pH and NHE-1 activity characteristic in low passage (immortalized) and high passage (transformed) human primary keratinocytes transfected with the HPV16 genome^a

HPK1A passage	Resting pHi	NHE activity	Δ pHi with DMA
Early	7.04 \pm 0.03 (7)	5.24 \pm 0.86 (4)	-0.05 \pm 0.006 (3)
Late	7.23 \pm 0.05 (7)**	11.86 \pm 3.91 (4)**	-0.18 \pm 0.017 (3)**

^a Values are mean \pm SE for the number of measurements indicated in the parentheses. NHE activity is in mM H^+ min^{-1} . Δ pHi with DMA is the change in resting pHi in cells in perfusion with 135 mM Na^+ ringer after the application of 2 μ M DMA. This acidification was reversible in both early and late passage HPK1A cells. ** $P < 0.001$.

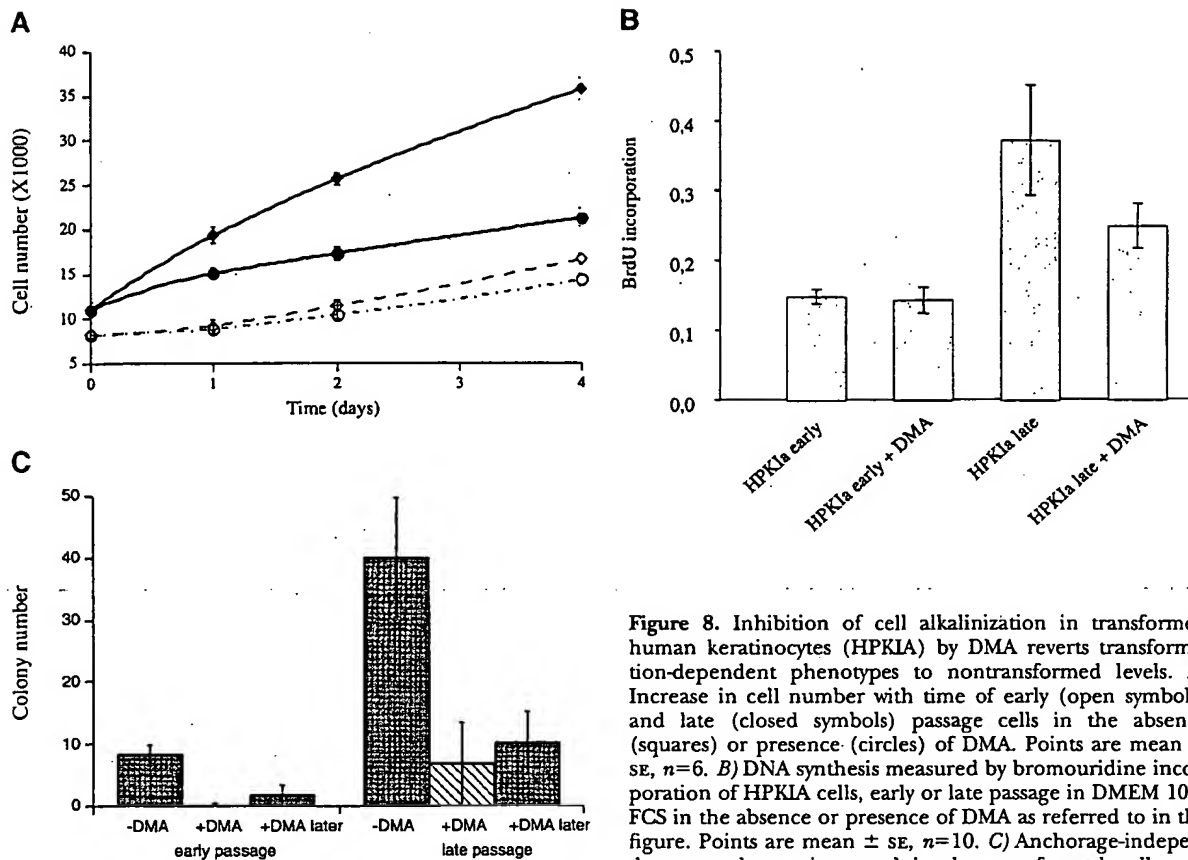


Figure 8. Inhibition of cell alkalization in transformed human keratinocytes (HPK1A) by DMA reverts transformation-dependent phenotypes to nontransformed levels. *A*) Increase in cell number with time of early (open symbols) and late (closed symbols) passage cells in the absence (squares) or presence (circles) of DMA. Points are mean \pm SE, $n=6$. *B*) DNA synthesis measured by bromouridine incorporation of HPK1A cells, early or late passage in DMEM 10% FCS in the absence or presence of DMA as referred to in the figure. Points are mean \pm SE, $n=10$. *C*) Anchorage-independent growth was increased in the transformed cells and

blocked by DMA treatment added either at the time of seeding or after colonies were allowed to grow for 1 wk (+DMA later). Number of colonies after 3 wk growth in 0.25% soft agar in early or late passage cells plus or minus DMA. Bars are mean \pm SE, $n=3$.

in nude mice. In line with the reported inhibition by amiloride analogs of the reinitiation of growth in quiescent cells without affecting steady-state growth (39), DMA inhibited only the component of growth stimulated by transformation. The abrogation of development of these transformed phenotypes is not due to an unspecific effect of DMA as decreasing of pH_i at nontransformed levels by acidification of culture medium or clamping of pH_i with the proton ionophore, tributyltin bromide, was also able to block the development of the transformed phenotypes.

Our data also demonstrate that alkalization is not simply a consequence of viral protein expression. In human keratinocytes the expression of HPV16 E6 and E7 genes is not sufficient to induce alkalization (low passage immortalized HPK1A cells). Since E6 and E7 associate with and inactivate the tumor suppressor proteins p53 and pRb, respectively, it is possible to conclude that alkalization is not dependent on pathways that are regulated by these two cellular proteins. pRb neutralization by HPV16 E7 leads to activation of the members of the E2F family, which in turn activate the transcription of positive

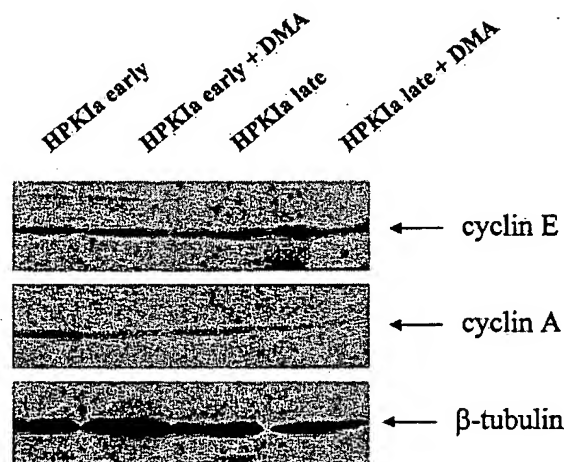


Figure 9. Inhibition of cell alkalization in transformed human keratinocytes (HPK1A) by DMA has no effect on endogenous levels of cyclin E and cyclin A. 5 μ M DMA was added to both early and late HPK1A passages and the expression of cyclin A and cyclin E was measured by Western blot analysis. Anti β -tubulin antibody was used to confirm that an equal amount of total proteins was loaded in each case.

cell cycle regulator genes, e.g., cyclins E and A. Our findings support the idea that the alkalinization-dependent proliferation is not mediated by transcriptional activation of E2F-regulated genes. In transient transfection experiments, we observed that inhibition of NHE-1 by DMA does not alter the ability of E7 to activate the cyclin E promoter. Moreover, the endogenous levels of cyclin E and A do not vary in transformed HPK1A cells (late passage) when cultured in absence or in presence of DMA, even though they have a different intracellular pH. Thus, additional cellular pathways are affected by the increase of pH_i to stimulate proliferation.

Cancer cells are characterized by an increased rate of glucose consumption metabolized predominantly by glycolysis under aerobic conditions (40, 41). This elevated rate has been considered to constitute the predominant metabolic component in highly malignant tumors (42), but little is known about its relative contribution to energy metabolism and growth in cells that have just undergone transformation. We show that activation of glycolysis (increased lactate production) is subsequent to and dependent on E7-induced alkalinization. Furthermore, we have observed that deoxyglucose treatment decreases the glycolysis to the levels of transformed HPK1A cells treated with DMA, but had little effect on cellular growth in comparison with the DMA treatment. Therefore, glycolysis appears not to play an impor-

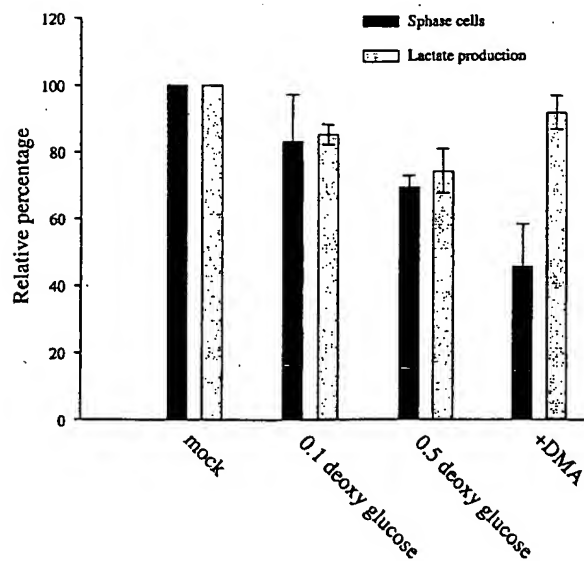


Figure 10. Stimulation of S-phase by transformation-dependent alkalinization is not linked to the activation of glycolysis. Late passage HPK1A cells were incubated with 0.1 or 0.5 mg/ml of 2'-deoxyglucose or 5 μ M DMA for 6 h and 24 h, respectively. The inhibition of glycolysis was measured as the production of lactate (gray bars) and the S-phase was monitored by BrdU incorporation (black bars). The results are shown as percentage inhibition of the untreated cells for each condition.

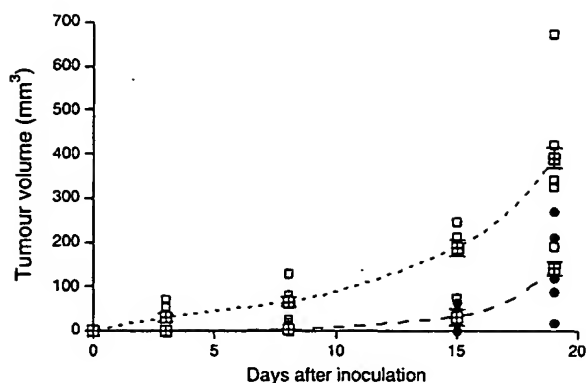


Figure 11. Induction of HPK1A tumors in nude mice is retarded by DMA treatment. Swiss athymic CD-1-nu/nu nude mice were injected subcutaneous (s.c.) with 10^7 HPK1A cells and half the mice were treated every other day with 15 μ g/gm body weight of DMA (circles) and the other half with vehicle (squares). Tumor size was measured at the indicated times after injection. The squares filled in with crosses represent the mean \pm standard error for each group on the indicated day.

tant role in the increase in growth rate observed on transformation in HPK1A cells.

In summary, our data demonstrate that alkalinization is an early and essential event in transformation and generates a permissive condition for the establishment and maintenance of transformation-associated phenotypes. The model system for cellular transformation presented in this study should provide a powerful tool for elucidating the signal transduction steps preceding alkalinization and the pH-dependent processes involved in the regulation of proliferation. [F]

We wish to thank Professor Harald zur Hausen for his continuous support to our work. We would like to thank professor M. Dürst for the kind gift of the HPK1A cell lines and Maria Elena Conte for her help in the laboratory. This work was supported in part by grant 97.00592.CT11 from the Italian National Research Council. M.A.F. was supported by a postdoctoral fellowship program of Alexander von Humboldt-Stiftung and S.C. by a PRAXIS XXI doctoral fellowship (Sub Programa Ciencia e Tecnologia do 2º Quadro Comunitário de Apoio).

REFERENCES

- Noel, J., and Pouyssegur, J. (1995) Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na⁺/H⁺ exchanger isoforms. *Am. J. Physiol.* 268, C283–C296
- Wakabayashi, S., Shigekawa, M., and Pouyssegur, J. (1997) Molecular physiology of vertebrate Na⁺/H⁺ exchangers. *Physiol. Rev.* 77, 51–74
- Hagag, N., Lacal, J. C., Graber, M., Aaronson, S., and Viola, M. V. (1987) Microinjection of ras p21 induces a rapid rise in intracellular pH. *Mol. Cell. Biol.* 7, 1984–1988
- Kaplan, D. L., and Boron, W. F. (1994) Long-term expression of c-H-ras stimulates Na-H and Na(+)-dependent Cl⁻/HCO₃⁻ exchange in NIH-3T3 fibroblasts. *J. Biol. Chem.* 269, 4116–4124

5. Rotin, D., Steele-Norwood, D., Grinstein, S., and Tannock, I. (1989) Requirement of the Na^+/H^+ exchanger for tumor growth. *Cancer Res.* 49, 205-211
6. Perona, R., and Serrano, R. (1988) Increased pH and tumorigenicity of fibroblasts expressing a yeast proton pump. *Nature (London)* 334, 438-440
7. Gillies, R. J., Martinez-Zaguilan, R., Martinez, G. M., Serrano, R., and Perona, R. (1990) Tumorigenic 3T3 cells maintain an alkaline intracellular pH under physiological conditions. *Proc. Natl. Acad. Sci. USA* 87, 7414-7418
8. Harguindey, S., Pedraz, J. L., Garcia Canero, R., Perez de Diego, J., and Cragoe, E. J., Jr. (1995) Hydrogen ion-dependent oncogenesis and parallel new avenues to cancer prevention and treatment using a H^+ -mediated unifying approach: pH-related and pH-unrelated mechanisms. *Crit. Rev. Oncog.* 6, 1-33
9. Shrode, L. D., Tapper, H., and Grinstein, S. (1997) Role of intracellular pH in proliferation, transformation, and apoptosis. *J. Bioenerg. Biomembr.* 29, 393-399
10. Mansur, C. P., and Androphy, E. J. (1993) Cellular transformation by papillomavirus oncoproteins. *Biochim. Biophys. Acta* 1155, 323-345
11. Dyson, N., Howley, P. M., Munger, K., and Harlow, E. (1989) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 243, 934-937
12. Davies, R., Hicks, R., Crook, T., Morris, J., and Vousden, K. (1993) Human papillomavirus type-16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. *J. Virol.* 67, 2521-2528
13. Hu, T. H., Ferril, S. C., Snider, A. M., and Barbosa, M. S. (1995) In vivo analysis of HPV E7 protein association with pRb, p107 and p130. *Int. J. Oncol.* 6, 167-174
14. Kouzarides, T. (1995) Transcriptional control by the retinoblastoma protein. *Semin. Cancer Biol.* 6, 91-98
15. Tommasino, M., and Crawford, L. (1995) Human papillomavirus E6 and E7: proteins which deregulate the cell cycle. *Bioessays* 17, 509-518
16. Morgenstern, J. P., and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18, 3587-3596
17. Paulus, W., Baur, I., Boyce, F. M., Breakefield, X. O., and Reeves, S. A. (1996) Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. *J. Virol.* 70, 62-67
18. Kraye-Pawlowska, D., Helmle-Kolb, C., Montrose, M. H., Krapf, R., and Murer, H. (1991) Studies on the kinetics of Na^+/H^+ exchange in OK cells: introduction of a new device for the analysis of polarized transport in cultured epithelia. *J. Membr. Biol.* 120, 173-183
19. Montrose, M. H., Friedrich, T., and Murer, H. (1987) Measurements of intracellular pH in single LLC-PK1 cells: recovery from an acid load via basolateral Na^+/H^+ exchange. *J. Membr. Biol.* 97, 63-78
20. Boron, W. F., and De Weer, P. (1976) Intracellular pH transients in squid giant axons caused by CO_2 , NH_3 , and metabolic inhibitors. *J. Gen. Physiol.* 67, 91-112
21. Roos, A., and Boron, W. F. (1981) Intracellular pH. *Physiol. Rev.* 61, 296-434
22. Watson, A. J., and Montrose, M. H. (1994) Rapid computerized analysis of Na^+/H^+ exchange flux. *Biochim. Biophys. Acta* 1193, 316-322
23. Belt, J. A., Thomas, J. A., Buchsbaum, R. N., and Racker, E. (1979) Inhibition of lactate transport and glycolysis in Ehrlich ascites tumor cells by bioflavonoids. *Biochemistry* 18, 3506-3511
24. Tedone, T., Correale, M., Paradiso, A., and Reshkin, S. J. (1996) Differential responsiveness of proliferation and cytokerauin release to stripped serum and oestrogen in the human breast cancer cell line, MCF-7. *Eur. J. Cancer* 32, 849-856
25. Brandt, R. B. (1982) Determination of D-lactate in plasma. *Methods Enzymol.* 89, 35-40
26. Chen, C., and Okayama, H. (1987) High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7, 2745-2752
27. Botz, J., Zerfass-Thome, K., Spitkovsky, D., Delius, H., Vogt, B., Eilers, M., Hatzigeorgiou, A., and Jansen-Dürr (1996) Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. *Mol. Cell. Biol.* 16, 3401-3409
28. Reshkin, S. J., Pezzetta, A., Albarani, V., Schittulli, F., and Paradiso, A. (1995) Characterization of a (3)H-GnRH method for the measurement of GnRH binding sites in human breast cancer and breast cancer cell lines. *Int. J. Oncol.* 7, 371-375
29. Tommasino, M., Adamczewski, J. P., Carloti, F., Barth, C. F., Manetti, R., Contorni, M., Cavalieri, F., Hunt, T., and Crawford, L. (1993) HPV16 E7 protein associates with the protein kinase p38CDK2 and cyclin A. *Oncogene* 8, 195-202
30. Banks, L., Edmonds, C., and Vousden, K. (1990) Ability of the HPV16 E7 protein to bind Rb and induce DNA synthesis is not sufficient for efficient transforming activity in NIH3T3 cells. *Oncogene* 5, 1383-1389
31. Phelps, W. C., Münger, K., Yee, C. L., Barnes, J. A., and Howley, P. M. (1992) Structure-function analysis of the human papillomavirus type 16 E7 oncoprotein. *J. Virol.* 66, 2418-2427
32. Siczkowski, M., Davies, J. E., and Ng, L. L. (1994) Activity and density of the Na^+/H^+ antiporter in normal and transformed human lymphocytes and fibroblasts. *Am. J. Physiol.* 267, C745-C752
33. Barry, M. A., Reynolds, J. E., and Eastman, A. (1993) Etoposide-induced apoptosis in human HL-60 cells is associated with intracellular acidification. *Cancer Res.* 53, 2349-2357
34. Rozhin, J., Sameni, M., Ziegler, G., and Sloane, B. F. (1994) Pericellular pH affects distribution and secretion of cathepsin B in malignant cells. *Cancer Res.* 54, 6517-6525
35. Li, J., and Eastman, A. (1995) Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification. Role of the Na^+/H^+ -antiport. *J. Biol. Chem.* 270, 3203-3211
36. Durst, M., Seagon, S., Wanschura, S., zur Hausen, H., and Bullerdiek, J. (1995) Malignant progression of an HPV16-immortalized human keratinocyte cell line (HPK1A) in vitro. *Cancer Genet. Cytogenet.* 85, 105-112
37. Maidorn, R. P., Cragoe, E. J., Jr., and Tannock, I. F. (1993) Therapeutic potential of analogues of amiloride: inhibition of the regulation of intracellular pH as a possible mechanism of tumour selective therapy. *Br. J. Cancer* 67, 297-303
38. Yamagata, M., and Tannock, I. F. (1996) The chronic administration of drugs that inhibit the regulation of intracellular pH: in vitro and anti-tumour effects. *Br. J. Cancer* 73, 1328-1334
39. L'Allemain, G., Franchi, A., Cragoe, E., Jr., and Pouyssegur, J. (1984) Blockade of the Na^+/H^+ antiport abolishes growth factor-induced DNA synthesis in fibroblasts. Structure-activity relationships in the amiloride series. *J. Biol. Chem.* 259, 4313-4319
40. Dang, C. V., and Semenza, G. L. (1999) Oncogenic alterations of metabolism. *Trends Biochem. Sci.* 24, 68-72
41. Webb, S. D., Sherratt, J. A., and Fish, R. G. (1999) Mathematical modelling of tumour acidity: regulation of intracellular pH. *J. Theor. Biol.* 196, 237-250
42. Bustamante, E., Morris, H. P., and Pedersen, P. L. (1981) Energy metabolism of tumor cells. Requirement for a form of hexokinase with a propensity for mitochondrial binding. *J. Biol. Chem.* 256, 8699-8704

Received for publication January 26, 2000.

Revised for publication May 9, 2000.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.